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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 93/14189
C12N 5/00, G01N 33/48, 37/00	A1	(43) International Publication Date:	22 July 1993 (22.07.93)

(21) International Application Number: PCT/US93/00083

(22) International Filing Date: 7 January 1993 (07.01.93)

(30) Priority data:
07/823,933 21 January 1992 (21.01.92) US

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(81) Designated States: AU, BB, BG, BR, CA, FI, HU, JP, KP, KR, LK, MG, MW, NO, PL, RO, RU, SD, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR DETERMINING CYTOLYTIC T CELL PRECURSORS

(57) Abstract

The invention relates to a method for determining precursors of cytolytic T cells and the frequency of these precursors, where the cytolytic T cells which develop therefrom are specific to antigens associated with or characteristic of the tumors. The method involves contacting peripheral blood monocyte cells (PBMC) containing sample to a source of antigen characteristic of or associated with the tumor, such as non viable tumor cells. The PBMCs, if precursors are contained therein, react and cytolytic T cells develop. These can then be determined by assaying for lysis of tumor cells introduced to the mixture. The methodology can be used for monitoring patient response to various therapeutic regimes.

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METHOD FOR DETERMINING CYTOLYTIC T CELL PRECURSORS

FIELD OF THE INVENTION

This invention relates to methods for determining precursors of cytolytic T cells specific for antigens characteristic of or specific to tumors. More particularly, it relates to a non-obvious application and modification of the "limiting dilution" assay to determine the precursors discussed above. The applications include the ability to monitor therapeutic regimes and subject response, to particular treatments, including immune responses.

BACKGROUND AND PRIOR ART

The ability to measure and assay events that occur in an immunological response is of great interest and importance in studying the course of a pathological condition or disease. Where a T-cell response is involved, of course, measurement and study of T cells is of concern.

T cells constitute a mixture of various types of lymphocytes, including cytolytic T cells, or "CTLs" as these will be referred to hereafter. CTLs interact with a molecule presented by their target cell via the T cell receptor. Following the interaction, the CTL causes the target cell to lyse.

The actual target recognized by the CTL is frequently referred to as an antigen, and this term will be used hereafter.

Prior work has established that various tumors can and do present antigens which can be the subject of a T-cell mediated response, leading to and rejection. Early work by Prehn et al., J. Nat. Canc. Inst. 18: 769-778 (1957); Klein et al., Canc. Res. 20: 1561-1572 (1960); Old et al., Ann. N.Y. Acad. Sci. 101:
80-106 (1962); Kripke et al., J. Nat. Canc. Inst. 53: 1333-1336 (1974), and Van Pel et al., J. Exp. Med. 157: 1992-2001 (1983) have established that the antigens expressed by these cells, now collectively referred to as "tumor rejection antigens", exist on murine tumors induced by viruses,

chemicals, and ultra-violet radiation, as well as on spontaneous tumors. It has also been shown that, in some tumor systems, these tumor rejection antigens induce cytolytic T cell responses and highly specific CTLs, directed against murine tumor rejection antigens have been isolated. See Brunner et al., J. Immunol. 124: 1627-1634 (1980). The target antigen of these CTLs is relevant for recognition by a syngeneic host, since tumor cells which escape partial immune rejection in vivo have been found to have masked or to have lost the antigen CTLs recognize. See Uyttenhove et al., J. Exp. Med. 157: 1040-1052 (1983). Adoptive transfer with cloned CTLs can eradicate tumor cells in animals bearing large tumors. See Kast et al., Cell 59: 603-614 (1989). The murine tumor P815 encodes a tumor rejection antigen, the gene for which has been identified and isolated, as per Van de Eynde et al., J. Exp. Med. 173: 1373-1384 (1991). It was found that this gene is identical to one that can be expressed by normal mouse cells, but has little or no expression in normal adult mouse tissues.

Studies extended to humans have found that mixed lymphocyte tumor cell cultures ("MLTCs") frequently generate responder lymphocytes which lyse autologous tumor cells without lysing natural killer ("NK") cells, autologous EBV transformed B cells or autologous fibroblasts. See Anichini et al., Int. J. Cancer 35: 683-689 (1985). The response has been studied for melanomas in MLTC, using peripheral blood monocyte cells (PBMCs) or tumor infiltrating lymphocytes (TILs) as reported by Mukherji et al., J. Exp. Med. 158: 240-245 (1983); Knuth et al., Proc. Natl. Acad. Sci. 86: 2804-2808 (1989); Herin et al., Int. J. Canc. 39: 390-396 (1987); Topolian et al., J. Clin. Oncol. 6: 839-853 (1988). MTLCs derived from PBMC generally contain responder cells which exert lysis on both tumor cells and on NK targets, when analyzed after two weeks of culture. After an additional two or three weeks, lytic activity on tumor cells increases, and that on NK targets disappears. See Herin et al., supra. It has been possible to derive CTLs from MLTC responders which seem to be completely

specific for tumors. The antigens recognized on tumor cells by these CTLs do not appear to be cultural artifacts, as they have been found to be present on fresh metastatic tumor tissue cells as well. See Mukherji, supra. Herin, supra; Knuth, supra. Panels of autologous CTLs permitted identification of four different stable antigens on human melanoma. See Van den Eynde et al., Int. J. Cancer 44: 634-640 (1989). Analysis of 70 additional CTLs from the patient on which the antigens were identified shows that all major stable antigens recognized by CTLs were in this set of four.

Assessment of the role of CTLs specific to tumor cells requires an ability to assay the frequency of their precursors in patients. This would permit the evaluation of, e.g., therapy protocols such as immune therapy approaches, and how these are affecting the CTL precursors (CTL-Ps), if at all.

The classic way to determine components of T cells is via a limiting dilution assay. This well known technique is described, e.g., by Sharrock et al., Immunol. Today 11(8): 281-285 (1990), the disclosure of which is incorporated by reference. These assays are designed to determine the frequency of a particular type of cell in a mixed population. A particular response is studied, in a number of different samples, and negative responses are used for further calculations. Negative responses are used because it is not immediately possible to know if a positive response can be associated with a particular cell. Essentially, a group of test cells, the "responder cells" are mixed with a second group, the "stimulator cells", and any reaction is then studied. The responder cells are used in a known or defined number, which can vary determining on the particular protocol being used. These assays have been carried out for CTLs, as reported by Sharrock, who discuss the importance of choice of an appropriate target cell, generally blast cells stimulated by concanvilin A or phytohemagglutinin. Epstein Barr virus transformants can also be used, if mixed with other targets. The technique, while useful, is described as being in its infancy by Sharrock et al. Thisreference does teach the use

of PBMCs as responder cells, but shows a limited number of choices as stimulators.

It has now been found that one can in fact assay for CTL-P cells which are specific to tumor antigens. This type of assay is not shown by the art, and is not suggested by the references which constitute the prior art. Thus, a method for assaying for CTL-Ps using a limiting dilution assay is the subject of the invention. The applications of this include the monitoring of subject response to therapeutic treatments, as explained infra.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a depiction of lytic patterns and criteria for their classification.

Figure 2 presents levels of tumor cell lysis via limiting dilution microcultures and Poisson analysis of data.

Figure 3 shows tumor cell lysis via limiting dilution, when competing K562 is added.

Figure 4 shows results obtained for frequencies in anti-tumor lytic effectors, in presence and absence of K562.

Figure 5 presents frequencies of lytic effectors among PBMCs, $CD4^+$ and $CD8^+$ cells.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The examples that follow use different reagents and cells in a variety of contexts. To simplify the presentation, certain explanations are provided here.

When "medium" is used, unless otherwise noted, it is Iscove's medium, supplemented with 0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM L-glutamine and $5x10^{-5}$ 2-mercaptoethanol.

Human serum, abbreviated hereafter as "HS", refers to pooled types A, B and O serum obtained from healthy donors. The HS had been decomplemented by treatment at 56°C for 30 minutes, partially delipified via centrifugation (45 minutes, 17,000g) filtration and sterilization.

Fetal bovine serum is abbreviated as "FBS".

Interleukin-2 and Interleukin-4 are abbreviated as IL-2 and

IL-4, respectively. In the following experiments, the recombinant, human form of the interleukins was used, although other forms would be expected to work as well. The concentrations used were 30 U/ml (IL-2), and 5 U/ml (IL-4) in all experiments. One unit/ml of IL-2 is defined as the concentration supporting half of maximal proliferation of CTLL-2 cell line. One unit/ml of IL-4 is that concentration which yields 50% maximal proliferation of human T cells previously treated with phytohemagglutinin-A ("PHA").

The cytokine "gamma interferon" is abbreviated as "IFN-γ".

EXAMPLE 1

Peripheral blood mononuclear cells (PBMCs) or purified subsets were obtained from patients. The patient samples were subjected to density gradient centrifugation, and were cryopreserved prior to use in the limited dilution assays described infra. To separate $CD4^+$ and $CD8^+$ T cells from these samples, flow cytometric sorting was used, employing labeled anti-Leu 3 antibody (fluorescein), and anti-Leu 2 antibody (phycoerythrin). The particulars of the sorting are not of special relevance to the invention, and purification of PBMCs, $CD4^+$ and $CD8^+$ can easily be carried out via standard methods. Sorted subpopulations were always more than 97.5% pure.

EXAMPLE 2

Limiting dilution assays were set up using various quantities of either PBMCs or the subsets described supra. The numbers ranged from about 200 to 10,000.

The particular amount, i.e., the defined number of PBMCs/subset chosen were seeded in 96 V-bottom microwells, together with 10^4 irradiated autologous tumor cells (i.e., tumor cells taken from the same patient as the PBMC source). The tumor cells had previously been used to establish cell lines, and the irradiated cells were taken therefrom. Irradiation was carried out using a Cs source (10000 rads).

Irradiated tumor cells served as stimulator cells. The medium, as defined supra used to culture the mixture was

supplemented with 10% HS and IL-4 as described supra. The wells were centrifuged for three minutes at 100g and incubated at 37°C in 8% CO_2. On the third day of incubation, IL-2 as described supra was added.

On the seventh day of culture, fresh medium (100 ul) was added, together with IL-2, IL-4, and 10^4 additional irradiated cells.

On the fourteenth day of culture, 100 ul of medium was discarded, the cells were transferred into flat bottom microwells, and IL-2, IL-4, and irradiated cells as above were added.

Over the course of culturing, the rate of proliferation of the CTL precursors in the sample were measured using radiolabeled thymidine and conventional methods. On those days, four aliquots of 40 ul of the cells were transferred into microwells and lytic activity was assayed. At the same time, the remaining samples were restimulated by adding 160 ul of medium, again containing the described IL-2, IL-4, and irradiated cells. At least 100 cultures were carried out for each number PBMC.

Stimulation of the type described supra led to proliferation of sufficient cells to allow assay of lytic activity against tumor cell.

EXAMPLE 3

To determine the lytic activity of the proliferated cells, these were tested with autologous tumor cells in the manner described below.

The autologous tumor cells to be used were preincubated for 48 hours in medium containing 50 U/ml IFN-γ. This enhances expression of histocompatibility molecules and adhesion molecules. When non-active natural killer cells ("K562", discussed infra) were added, these were not so treated. IFN-γ does not modify susceptibility of these cells to lysis via NK-like effectors.

The tumor cells were labeled via suspending them at 10^7 cells/ml in medium supplemented with 10% HS, and incubated at 37°C for 60 minutes, using 200m ^{51}Cr Ci/ml. The labeled cells

were washed three times with medium and 2% HS, and were then suspended in medium at 10^4 cells/ml. Following this, 60 ul of medium augmented with 2% HS or with an additional $5x10^4$ K562 cells/well were added to wells containing the previously stimulated cells. After one hour of this incubation (37°C), 1000 tumor cells were added per well in 100 ul of medium. The mixtures were centrifuged (4 minutes, 200g) and then incubated for 4-5 hours at 37°C in 8% CO_2 atmosphere. Aliquots of supernatant (100 ul) were collected and ^{51}Cr specific release calculated. The following formula was used:

$$\%RELEASE = (ER-SR)x100(MR-SR)$$

Where: ER is experimental ^{51}Cr release, SR = spontaneous release (i.e., release by cells incubated in medium alone), and MR the "maximal release", i.e., that obtained by incubating the tumor cells in 0.15% TRITON X-100. SR never exceeded 15% of MR.

Figure 2 shows results secured using samples taken from two patients (LB-33 & LB-30), using varied numbers of PBMCs and the percentage lysis of melanoma cells taken from the patient. High degrees of lysis were observed in many microcultures. In control cultures, i.e., those where no tumor cells, and hence no antigen was presented, there was no lysis.

As the number of PBMCs decreased, the fraction of positive microcultures also decreased, but the level of lysis did not decrease more than expected.

Analysis of the lysis pattern showed that a clear bimodal distribution allowing easy discrimination between positives and negatives did not exist. This is consistent with an earlier observation that different effector cells display different rates of proliferation, thus affecting the degree of lysis.

When the percentage of microcultures devoid of lytic activity is plotted as a function of PBMC number and using zero order terms of Poisson distribution, the logarithm of the fraction of negative wells decreased linearly when the quantity of PBMC increased. Figure 2 shows that this is true, regardless of whether 5% or 10% lysis was chosen as threshold

for positivity.

EXAMPLE 4

In the past, stable lytic clones derived from responder cells have been reported to include anti-tumor CTLs, which show little or no lysis of the cell line K562, which is a natural killer (NK) cell target, as well as other clones which do lyse K562. (K562 is a human chronic myelogenous leukemia cell line, freely available from the American Type Culture Collection, e.g., under Accession Number ATCC CCL 243). This cell line has been described, e.g., at J. Nat. Canc. Inst. 59: 77-83 (1977), as a highly sensitive in vitro target in NK assays. The K562 specific lytic clones are referred to as "NK like", and it was important to determine if these were present.

To do this, the limited dilution materials described supra were assayed for affect on K562, the NK target. Assays were run, in both the absence and presence of a 50 fold excess of non-viable K562 relative to the number of tumor cells in the sample. The assays used the chromium release methodology discussed supra. The result showed that stimulated PBMC samples fall into one of four patterns:

1. Some microcultures lyse tumor cells, but do not lyse K562. In such cultures, competition by unlabeled K562 never significantly reduced the tumor cell lysis.

2. Both tumor cells and K562 were lysed. In competition with "cold" K562, the lysis of labeled K562 was nearly abolished, but tumor cell lysis was not. Such cultures show a mixture of tumor specific, and NK like clones.

3. Both tumor and K562 cells lysed, where competition with cold K562 abolishes all lysis. Such cultures appear to contain at least one NK like clone.

4. Lysis of only K562. Such cultures clearly contain NK like clones, but not tumor specific cells.

The sum of "1", "2" and "3" corresponds to the total amount of anti-tumor lytic clones as described supra. Patterns "1" and "2" indicate anti-tumor CTLs, and can be used to determine

the frequency of CTL precursors. Table 1 summarizes the classification into one of the four types listed supra. The abbreviation "LDA" used therein stands for "limiting dilution assay".

In the presence of competing K562, a decrease in PBMC number did not reduce tumor cell lysis more than what would be expected from loss of cultures containing multiple CTL clones. These results are shown in Figure 3.

Table 1. Patterns of lysis observed in anti-tumoral LDA experiments.

Patient	PBMC/ well	Targets	% lysis by LDA microcultures:																					
LB-33	2295	TUM	22	3	60	50	55	30	42	0	50	50	42	47	37	21	28	44	38	30	54	38	3	
		TUM+K562	22	0	68	61	66	26	43	0	58	66	43	62	34	14	45	40	44	31	61	38	10	
		K562	48	54	53	49	31	53	63	56	47	10	42	43	41	48	30	56	0	49	39	50	45	
		K562+K562	0	1	0	0	0	1	1	5	0	0	0	0	0	0	0	1	0	0	0	0	0	
			II	IV	II	II	II	II	II	IV	II	II	II	II	II	II	II	II	I	II	II	II	IV	
	1020	TUM	25	0	16	44	33	0	34	40	0	18	13	47	0	0	0	40	10	0	0	4	1	
		TUM+K562	30	0	16	46	42	0	33	36	0	12	12	60	0	0	1	39	14	0	0	10	3	
		K562	26	10	40	15	39	54	40	48	54	55	39	2	52	50	49	56	7	82	30	48	0	
		K562+K562	0	0	0	0	0	0	0	1	0	6	0	0	0	0	0	0	0	0	0	2	0	
			II	0	II	II	II	IV	II	II	IV	II	III	I	IV	IV	IV	II	0	IV	IV	IV	0	
	453	TUM	38	23	0	0	1	0	0	32	0	18	22	49	14	0	0	0	0	0	38	2	18	
		TUM+K562	48	22	0	0	5	0	0	37	0	21	26	56	19	0	0	0	0	0	46	2	20	
		K562	0	51	26	65	50	0	19	56	0	0	0	1	0	18	0	0	4	0	37	59	0	
		K562+K562	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
			I	II	IV	IV	IV	0	IV	II	0	I	I	I	I	IV	0	0	0	0	II	IV	I	
	302	TUM	0	7	0	0	0	0	41	41	0	0	43	0	0	0	0	0	0	28	0	28	0	
		TUM+K562	0	1	0	0	0	0	0	54	0	0	54	0	0	0	13	0	0	36	0	37	0	
		K562	0	58	2	0	0	0	59	46	6	0	0	59	0	1	0	0	0	0	2	0	0	
		K562+K562	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
			0	IV	0	0	0	0	III	II	0	0	I	IV	0	0	0	0	0	I	0	I	0	
LB-34	988	TUM	12	0	14	0	7	0	71	32	10	7	3	46	0	48	57	0	11	18	1	8	11	
		TUM+K562	0	0	0	0	0	0	57	20	0	0	0	42	0	41	19	0	3	3	0	0	10	
		K562	85	71	77	76	0	58	73	39	77	38	63	0	16	62	2	21	42	64	4	29	80	
		K562+K562	3	2	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
			IV	IV	III	IV	0	IV	II	II	IV	III	IV	I	IV	II	I	IV	III	III	0	III	IV	
	439	TUM	0	0	40	0	0	0	18	0	5	0	1	2	15	41	46	0	0	0	20	55	1	
		TUM+K562	0	0	0	0	0	3	0	0	0	0	0	0	0	54	2	0	0	0	20	38	0	
		K562	45	8	87	18	78	85	88	0	77	13	47	70	82	0	78	12	11	78	74	83	61	
		K562+K562	0	0	15	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
			IV	0	III	IV	IV	IV	III	0	IV	0	IV	IV	III	I	III	0	0	IV	II	II	IV	
LG-2	1053	TUM	48	54	5	26	51	13	19	4	12	52	52	57	33	24	51	27	16	26	8	42	24	
		TUM+K562	4	0	0	0	0	0	0	0	0	50	0	2	0	0	0	0	0	1	0	5	0	
		K562	87	77	80	82	86	36	77	78	75	77	80	74	83	80	83	82	70	75	64	86	75	
		K562+K562	3	12	0	3	10	0	3	2	1	0	6	24	2	2	9	5	1	7	0	0	2	
			III	III	IV	III	III	III	III	IV	III	II	III	III	III	III	III	III	III	III	IV	III	III	
	1053	TUM	8	27	18	17	18	29	14	31	52	7	37	22	12	38	10	34	20	47	2	7	3	
		TUM+K562	0	0	0	0	24	0	9	0	4	0	0	0	0	0	0	0	0	18	0	0	1	
		K562	11	81	81	77	25	81	66	85	83	33	86	84	72	83	79	85	71	88	46	56	29	
		K562+K562	3	5	1	2	1	3	0	8	5	1	1	3	0	5	1	2	3	6	2	2	1	
			0	III	III	III	II	III	III	III	III	III	III	III	III	III	IV	III	III	II	IV	IV	IV	
	1053	TUM	9	3	3	29	23	20	28	13	9	28	43	4	8	14	20	54	37	26	26	3	19	
		TUM+K562	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	0	0	0	
		K562	73	47	66	83	74	79	67	70	58	82	89	83	41	75	38	78	82	72	82	59	78	
		K562+K562	8	4	3	12	8	4	1	3	0	2	8	1	3	0	0	5	2	4	3	1	10	
			IV	IV	IV	III	III	III	III	III	III	III	III	IV	IV	III	II	III	III	III	III	IV	III	
	1053	TUM	8	64	6	6	24	15	1	14	42	12	24	17	4	28	43	46	11	60	34	14	6	
		TUM+K562	0	3	0	0	1	1	0	0	7	0	0	7	0	0	0	49	0	0	0	0	0	
		K562	71	80	82	21	58	78	7	66	82	66	79	30	23	66	78	69	62	98	81	68	85	
		K562+K562	0	21	4	1	1	3	0	8	5	4	1	0	0	6	3	2	3	14	3	4	4	
			IV	III	IV	III	III	III	0	III	III	III	III	II	III	III	III	II	III	III	III	III	IV	

EXAMPLE 5

The clones considered to be anti-tumor CTLs were examined for specificity. This was done by testing lytic cultures on various targets. Table 2, which follows, presents one set of these data, where PBMCs of a patient (LB-33) were tested against autologous tumor cells, K562, autologous PHA blasts, and tumor cells from patients LB-30 and LB-34. These cells were taken from cell line cultures as discussed supra. The lysis percentage was determined 28 days after setting up a lytic assay as described supra, using 1333 PBMCs per well. In the case of PHA-blasts, lysis was checked using autologous PBMCs activated for 10 days with 1000 U/ml of IL-2.

Table 2. Specificity of lysis by LDA microcultures.

Targets	Competing K562	% lysis by LDA microcultures: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Autologous Tumor	-	52	0	73	0	43	39	35	0	0	69	4	1	43	6	61	0	64	14	58	11	73	57	25	0
	+	49	0	75	0	28	7	53	0	0	63	1	1	33	6	63	0	77	5	68	5	80	72	22	0
K562	-	0	0	9	1	5	1	0	39	0	0	0	18	0	4	0	48	33	11	0	13	1	19	0	0
	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Autologous PHA blasts	-	0	1	3	2	1	0	0	0	0	0	0	6	0	0	0	5	0	0	2	1	6	9	3	0
	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	0	1	0	0	0
LB-30-MEL	-	5	0	2	0	0	1	0	1	0	0	0	0	1	0	0	1	2	2	1	0	0	0	2	0
	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
LB-34-MEL	-	14	0	0	0	0	0	0	0	0	7	0	0	0	0	0	1	2	0	0	1	0	0	0	0
	+	6	0	0	0	1	0	0	0	0	16	0	0	0	0	0	0	0	0	0	1	0	0	0	0

EXAMPLE 6

Frequency of anti-tumor CTL precursor cells in various patients was evaluated. Limiting dilution cultures were set up using PBMCs, as described supra, using all of the indicated materials. Lytic activity was measured on the day indicated in the panels of Figure 4 ("d20", e.g.), and frequency of lytic effectors calculated. Anti-tumor and total of all lytic effectors are presented.

The relationship between number of PBMCs added and logarithm of fraction of microcultures without anti-tumor CTLs appear linear. This supports the point raised supra, that tumor specific lysis appears to result from activity of single T lymphocyte clones. Large variations were observed in different patients, both in frequencies of all tumor cell lysing cells, and in precursors of anti-tumor specific CTLs. The ratios between these two frequently showed large differences as well.

EXAMPLE 7

Analysis was carried out to determine frequency of NK-like effector clones, as detected around day 20 of the limiting dilution assays, as well as anti-tumor CTL precursor frequencies. These are shown in following Table 3.

Table 3. Anti-tumoral CTL-P frequencies of different melanoma patients.

Patient	Exp.	Day	Frequencies of specific anti-tumor CTL-P	Frequencies of precusors of NK-like effector cells
LB-33	1+	20	1/2290	1/860
	2	18	1/1180	1/770
		22	1/1690	1/2560
		29	1/2310	1/8350
	3	20	1/1220	1/780
	4	18	1/910	1/1020
	5	20	1/1440	1/610
		28	1/2100	1/5090
LB-34		21	1/6310	1/910
LB-25		18	1/7120	1/250
MZ-2	1*	26	1/13400	1/980
	2	22	1/9640	1/2560
LB-30	1+	25	1/10800	1/420
	2	27	1/12300	1/2050
LB-17		31	1/17900	1/830
LG-2		20	1/33800	>1/400

This table shows activity against autologous tumor cells, both with and without non-viable K562. The microcultures of pattern "1" or "2" were used to estimate CTL precursor (CTL-P) frequencies, and patterns "2", "3" or "4" used to estimate NK-like effector cell frequencies.

It is noted that constant CTL-P frequencies were observed even though there was some decrease with time in the number of microcultures with activity. The reason for this is that under the conditions used, most CTL clones do not proliferate indefinitely, most probably because of the absence of feeder cells.

Cultures with NK-like activity decreased more severely between days 14 and 30, which is consistent with prior observations by Hérin et al., Int. J. Cancer 39: 390-396 (1987).

EXAMPLE 8

Prior experiments leading to isolation of anti-tumor CTL clones by limited dilution around day 15 showed both $CD4^+$ and $CD8^+$ cells, but the vast majority of anti-tumor CTLs were $CD8^+$. Studies were carried out to determine if this was because culture conditions favor production of $CD8^+$ cells. Using the sorting methodologies described supra, it was found that most CTL-Ps are $CD8^+$

Several features of the examples presented supra merit comment. First, the microcultures did not use feeder cells in the cultures. For many patients, it is difficult to obtain the autologous PBMCs that are desirable feeder cells in sufficient amounts. It was also desired to present a generally applicable limiting dilution assay. In experiments performed by the inventors and not reported herein, when autologous, irradiated PBMCs were used as feeder cells, an increase in anti-tumor CTL frequency was observed, but this was coupled to higher NK-like frequency.

The experiments using the NK inhibiting material, i.e., non-viable K562 cells permits distinction between anti-tumor CTLs and NK like effectors which also lyse tumor cells. The NK like effectors are inhibited by binding to the K562 cells,

which, since they are non viable, do not show ^{51}Cr release. Other methods to eliminate the effect of NK and/or NK like cells in the mixture, including other NK target cells, can also be used.

The frequencies of CTL-Ps specific for antigens characteristic of tumor cells ranged from 1/33000 to 1/900 PBMCs. The latter number, obtained with patient LB-33, may result from an autoimmune response against a tumor. The numbers observed should be compared to those secured by Borysiewicz et al., Eur. J. Immunol. 18: 269-275 (1988) of 1/5000-20,000 for CMV, by Schmid et al., J. Immunol. 140: 3610-3616 (1988) of 1/4000-8000 for HSV, or by Hickling et al., J. Virol. 61: 3463-3469 (1987), for varicella zoster of 1/1600-90000. Sharrock, Immunol Today 11: 281-286 (1990) discusses alloreactive frequencies of 1/500-500000.

The invention thus teaches a method by which cytotoxic T cell precursors (CTL-Ps) specific for antigens characteristic of tumor cells can be determined via a limiting dilution assay. In the methodology, a defined number of peripheral blood mononuclear cells, which contain the CTL-PS, are contacted to an antigen which is characteristic of, charge for, i.e., is particularly associated with, a tumor type or types. This results in a mixture of materials which is then cultured. The stimulation from the tumor antigen causes the CTL-Ps to develop into the actual cytolytic T cells. This development is monitored via monitoring the lysis of tumor cells either in or added to the mixture.

The number of PBMCs used may vary, but preferably somewhere between about 200 and about 10000 are used per assay. The PBMCs may be added in a mixed sample, or as a pure culture of PBMCs.

The antigen characteristic of the tumor is ideally added in the form of a tumor cell, generally non-viable. The tumor cell can be rendered non-viable via, e.g., irradiation. The tumor cells still present the antigen on their cell surfaces, and a proliferation linked reaction with CTL-Ps follows. The antigen can also be added, e.g., in pure form, but this is not

the preferred mode.

It is especially preferred to administer an autologous sample of tumor cells - i.e., tumor cells taken from the same subject as the PBMCs. Ideally, the cell sample is as pure as possible to prevent reaction between CTL-Ps not directed against tumor cells and their targets. This is not always possible, however, and thus it is preferred to treat the mixture to eliminate natural killer ("NK") like cells contained therein. The term "natural killer like" includes natural killer cells, as well as cells which function in the same or an equivalent manner. One approach to doing this is by adding, e.g., an NK inhibitor.

The lytic determination can be accomplished by means well known in the art, including the ^{51}Cr release method, described supra.

Desirably, when culturing the mixtures described herein, this is done without the use of feeder cells; however, this is not a requirement.

The ability to monitor CTL-Ps also enables one to monitor responses such as the immune response of a subject with respect to the subject's tumor. Changes in CTL-P levels are indicative of changes in the immune response, and serve an important diagnostic function in that changes over time can indicate a worsening or improvement in the subject's health.

Various modifications to the method described herein have been shown. Others will be clear to the skilled artisan and are not repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

We Claim:

1. Method for determining a precursor of a cytotoxic T cell specific for an antigen characteristic of a tumor, comprising:

(i) contacting a defined number of peripheral blood mononuclear cells (PBMCs) taken from a subject to an antigen characteristic of said tumor to form a mixture,

(ii) culturing said mixture under conditions favoring development of cytolytic T cells, specific for said antigen from said PBMCs, and

(iii) determining lysis of tumor cells added to said mixture by said cytolytic T cells as an indication of said precursor in said subject.

2. Method of claim 1, wherein said defined number ranges from about 200 to about 10000.

3. Method of claim 1, wherein said tumor specific antigen is a tumor rejection antigen.

4. Method of claim 1, comprising adding a sample containing tumor cells to said PBMCs to form said mixture, wherein said tumor cells are derived from the same subject as said PBMCs.

5. Method of claim 4, comprising treating said mixture to inactivate natural killer-like cells contained therein.

6. Method of claim 5, wherein said treating comprises adding a natural killer cell inhibitor to said mixture.

7. Method of claim 6, wherein said inhibitor is a non-viable cell which is a natural killer cell target cell.

8. Method of claim 7, wherein said natural killer cell target is K562.

9. Method of claim 1, comprising culturing said mixture in the absence of feeder cells.

10. Method for monitoring response of an individual to treatment of a tumor related condition, comprising:

(i) determining precursor cytolytic T cells formed by said subject which are specific for an antigen associated with said tumor in said subject at a first point in time,

(ii) determining precursor cytolytic T cells specific for said antigen associated with said tumor at a second point in time, and

(iii) comparing values obtained in (i) and (ii), wherein a difference therebetween is indicative of a change in said immune response.

11. Method of claim 10, comprising determining precursor cytolytic T cells by culturing a defined number of PBMCs taken from the individual with said tumor related condition with a tumor sample taken from said subject and determining lysis of said cells in said tumor sample as a measure of cytolytic T cells.

12. Method of claim 4, wherein said tumor cells are non-viable.

13. Method of claim 3, wherein said antigen is a tumor rejection antigen.

14. Method of claim 11, wherein said defined number PBMCs ranges from about 200 to about 10000.

15. Method of claim 11, wherein said tumor sample is a sample presenting a tumor rejection antigen.

FIG. 1

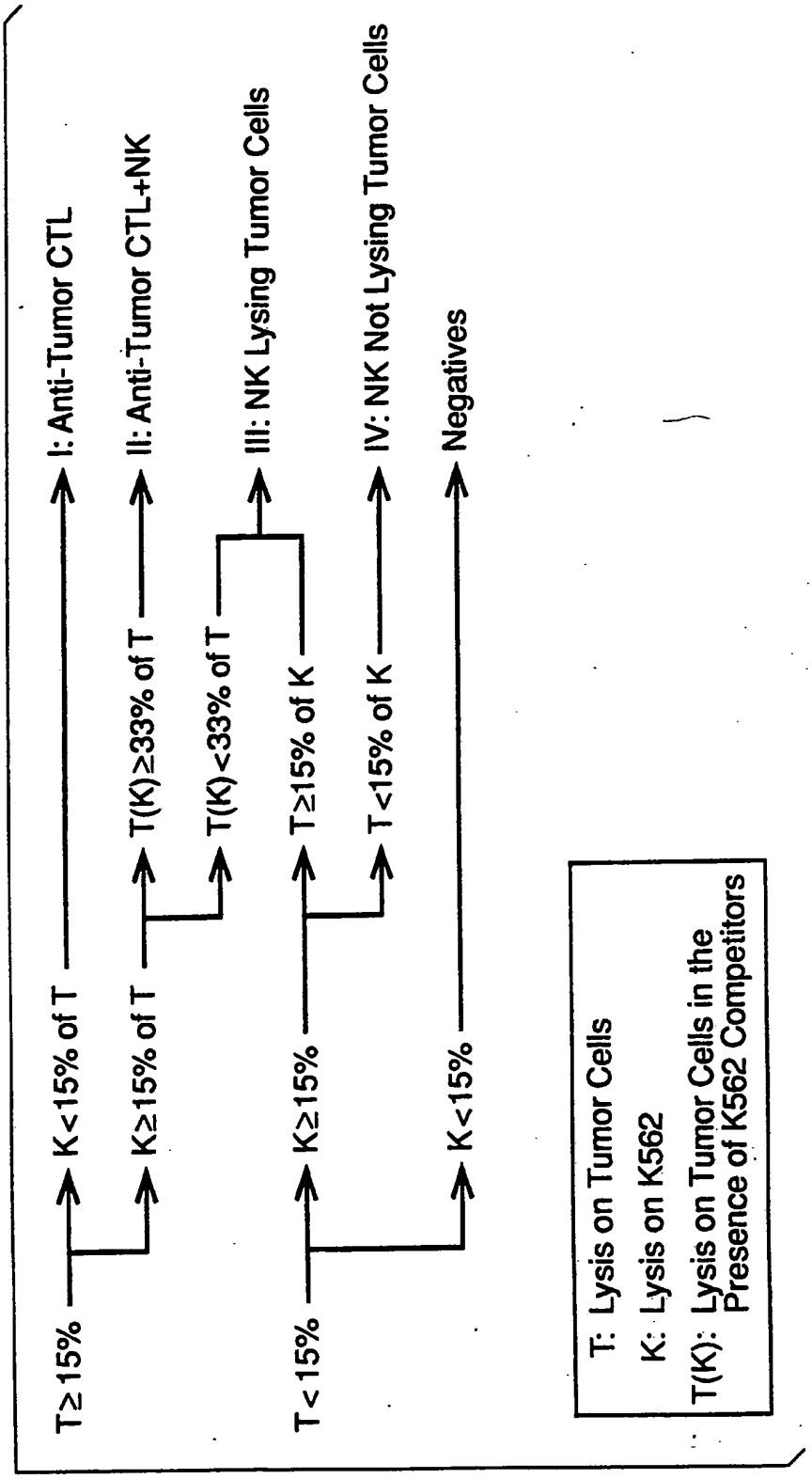

T≥15%
K<15% of T
I: Anti-Tumor CTL
K≥15% of T
T(K)≥33% of T
II: Anti-Tumor CTL+NK
T(K)<33% of T
III: NK Lysing Tumor Cells
T<15%
K≥15%
T≥15% of K
IV: NK Not Lysing Tumor Cells
T<15% of K
K<15%
Negatives
T: Lysis on Tumor Cells
K: Lysis on K562
T(K): Lysis on Tumor Cells in the Presence of K562 Competitors

FIG. 2B

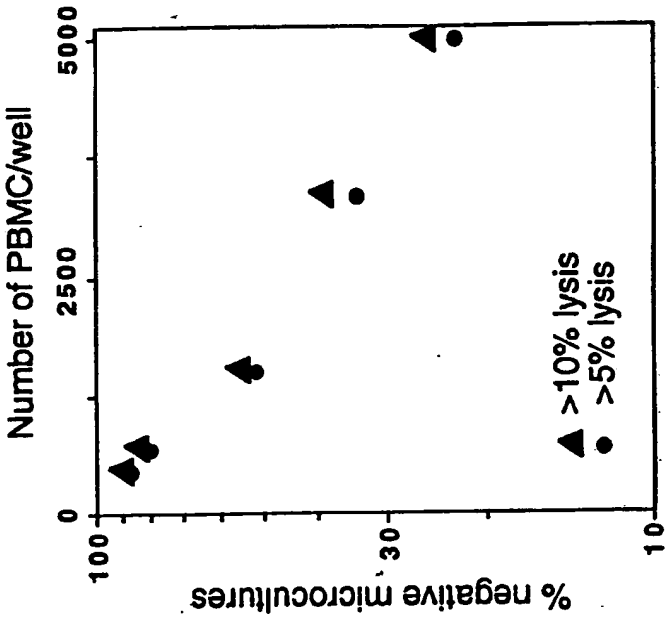

Number of PBMC/well
5000
2500
0
100
30
10
% negative microcultures
>10% lysis
>5% lysis

FIG. 2A

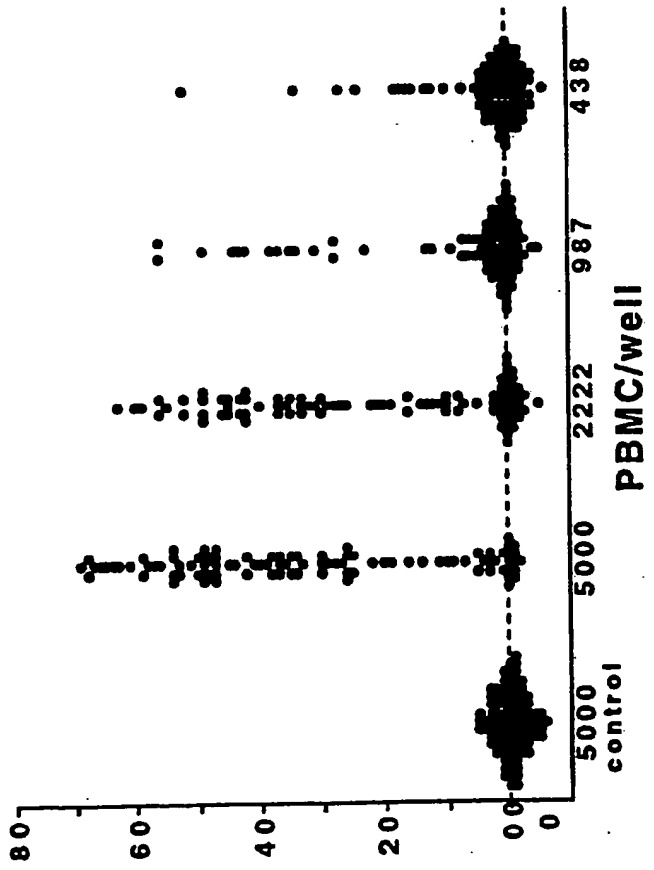

% lysis of tumor cells
80
60
40
20
00
0
5000 control
5000
2222
987
438
PBMC/well

FIG. 2D

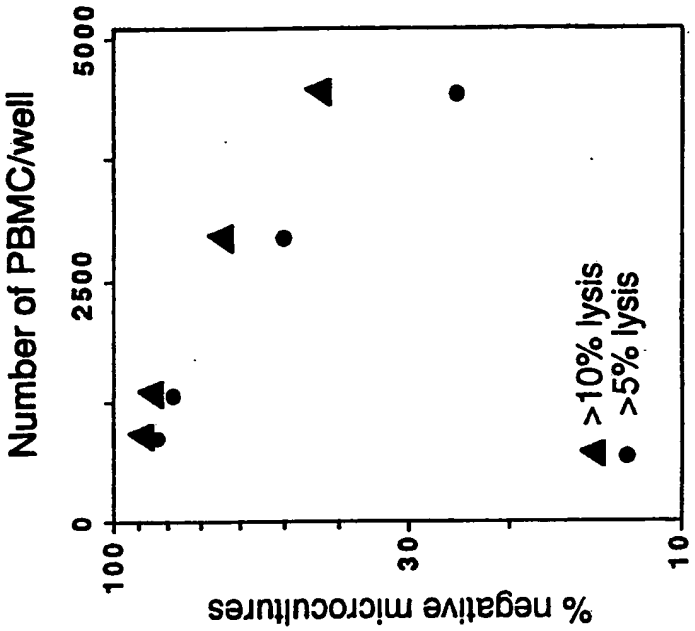

Number of PBMC/well
5000
2500
0
100
30
10
% negative microcultures
>10% lysis
>5% lysis

FIG. 2C

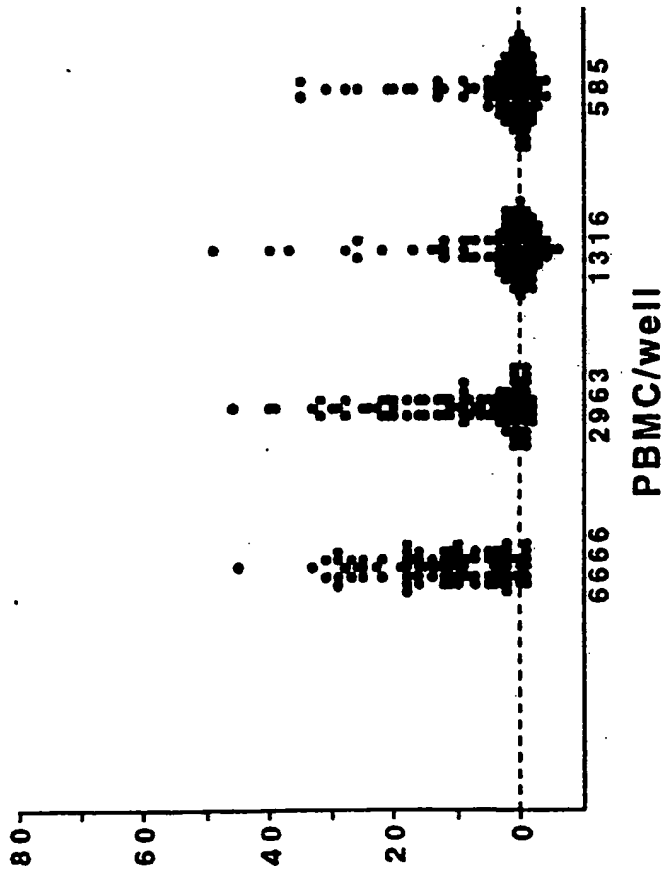

% lysis of tumor cells
80
60
40
20
0
6666
2963
1316
585
PBMC/well

FIG. 3A

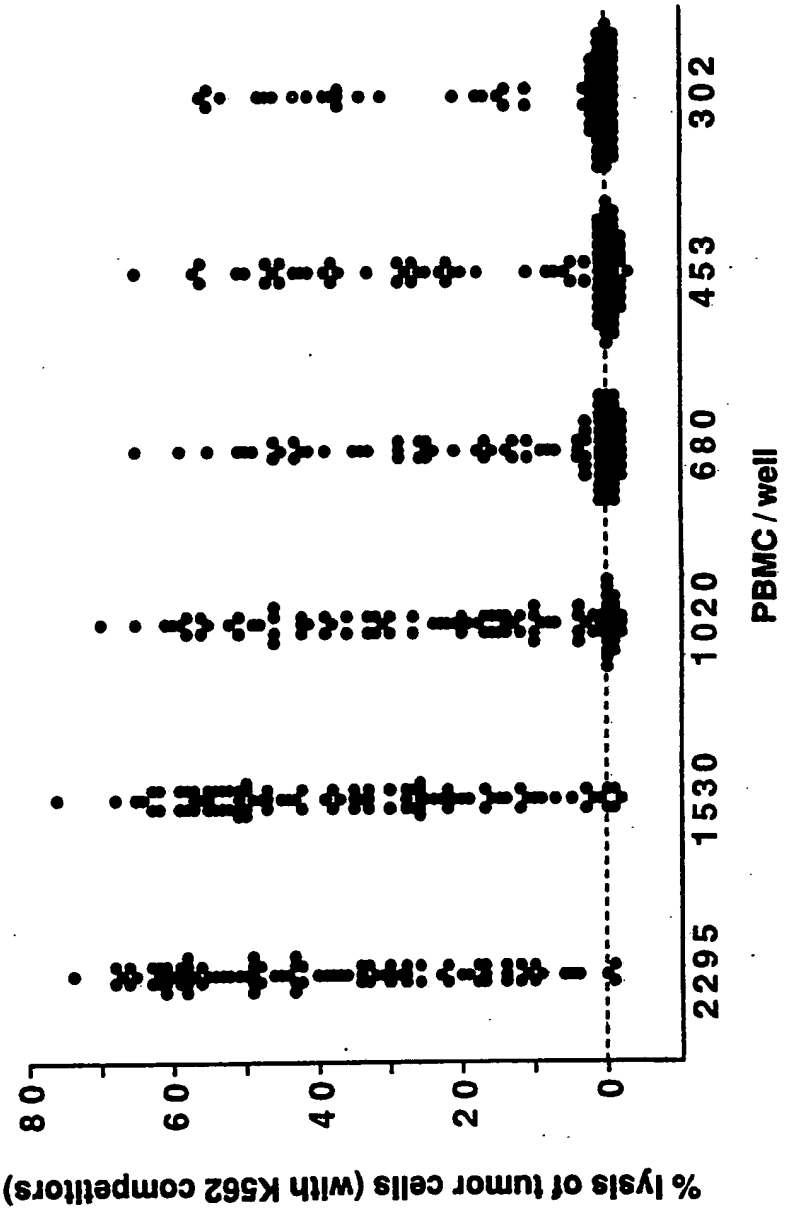
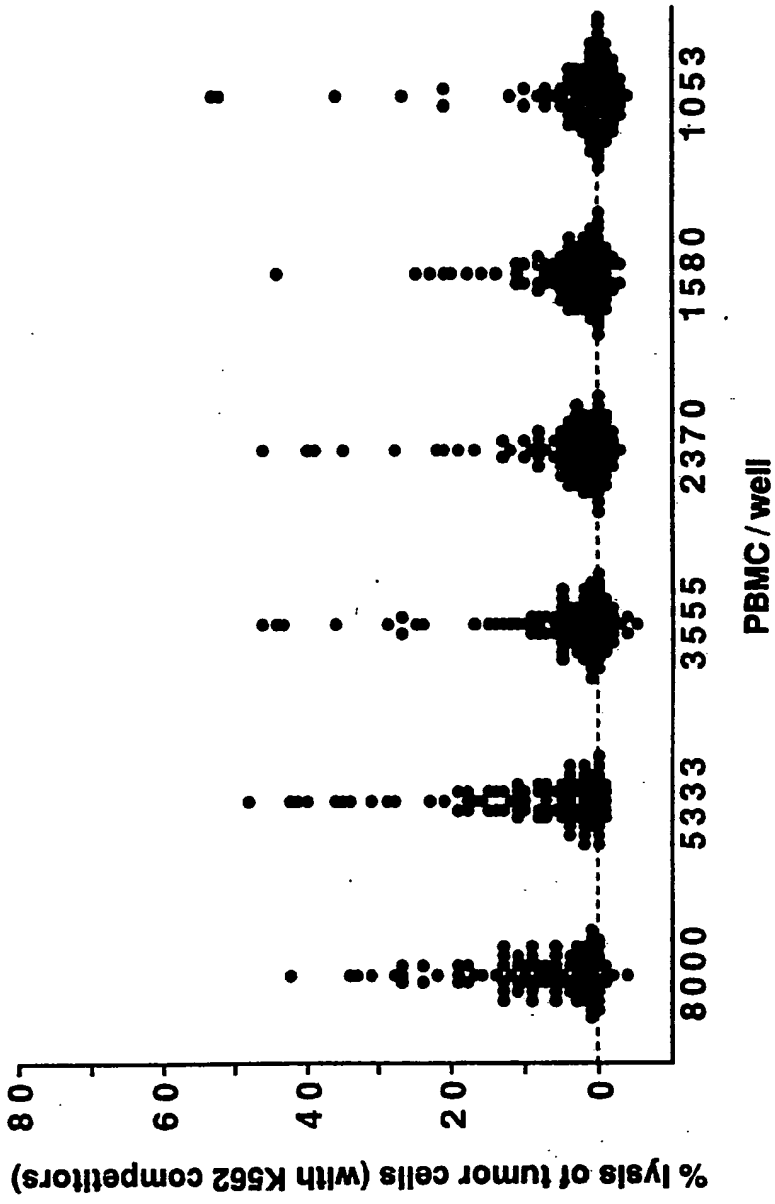

% lysis of tumor cells (with K562 competitors)
80
60
40
20
0
2295
1530
1020
680
453
302
PBMC / well

FIG. 3B


% lysis of tumor cells (with K562 competitors)
80
60
40
20
0
8000
5333
3555
2370
1580
1053
PBMC / well

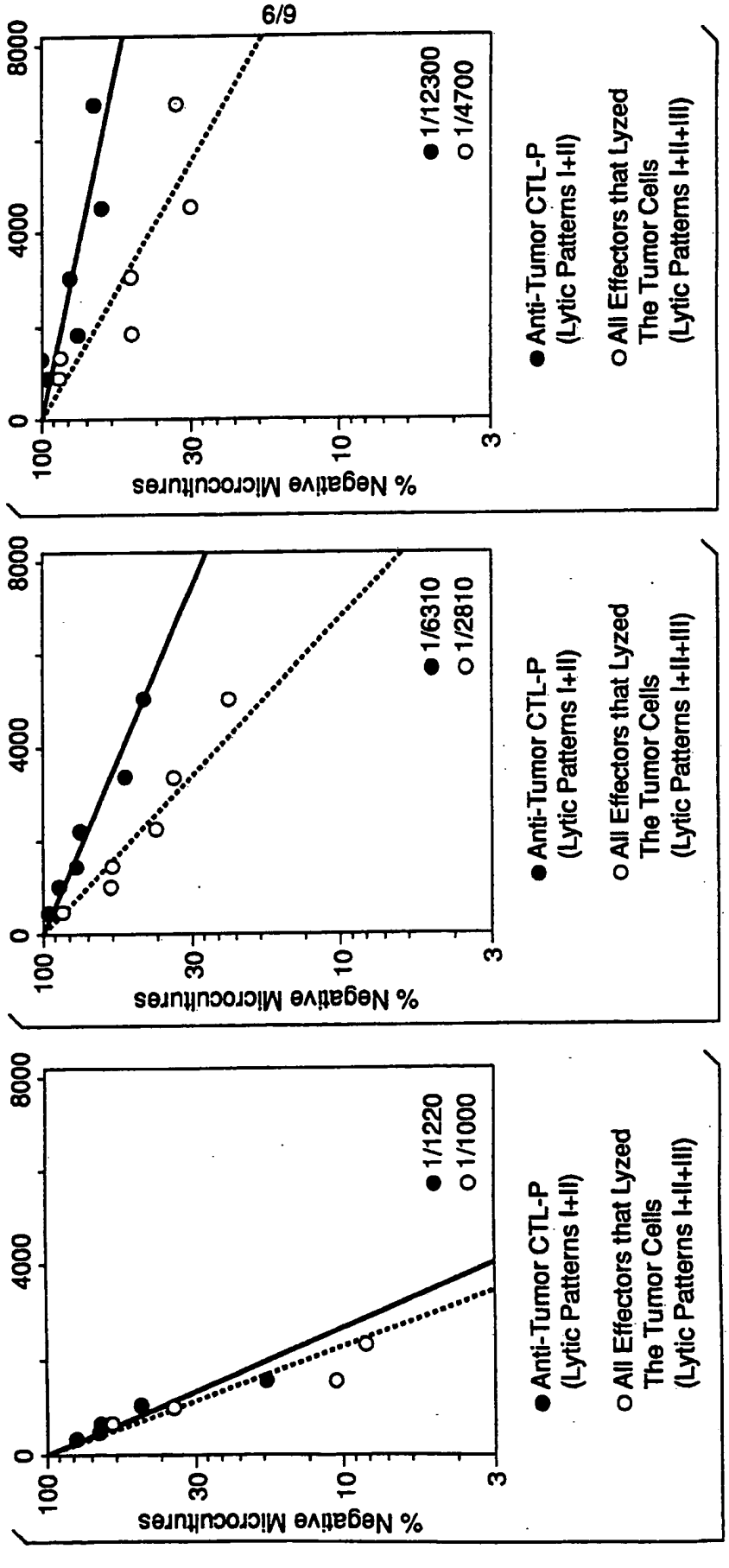


FIG. 4C

FIG. 4B

FIG. 4A

FIG. 4E

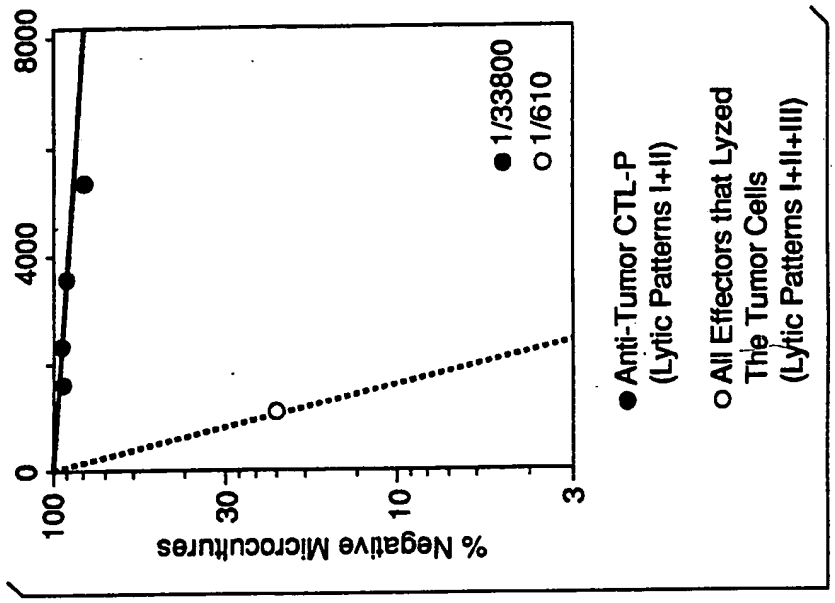

8000
4000
0
100
30
10
3
% Negative Microcultures
● 1/33800
○ 1/610
● Anti-Tumor CTL-P
(Lytic Patterns I+II)
○ All Effectors that Lyzed
The Tumor Cells
(Lytic Patterns I+II+III)

FIG. 4D

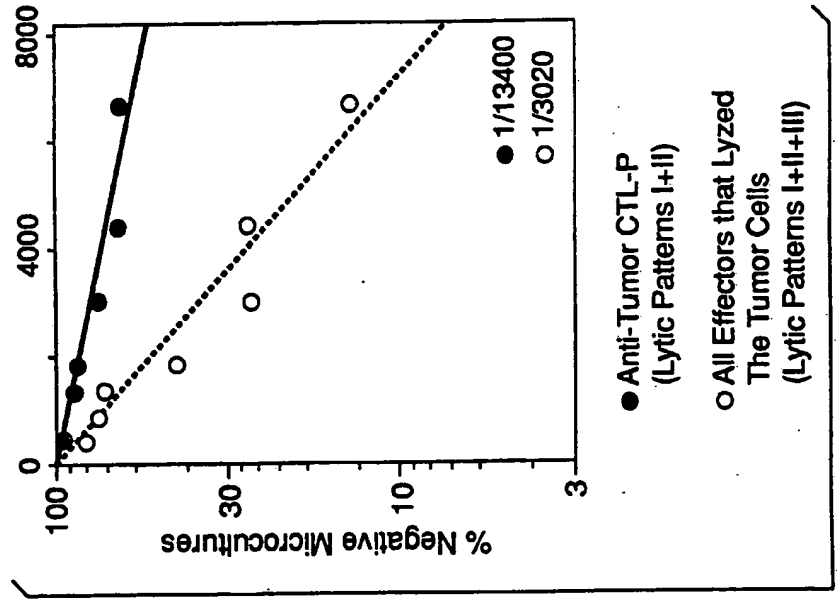

8000
4000
0
100
30
10
3
% Negative Microcultures
● 1/13400
○ 1/3020
● Anti-Tumor CTL-P
(Lytic Patterns I+II)
○ All Effectors that Lyzed
The Tumor Cells
(Lytic Patterns I+II+III)

FIG. 5A

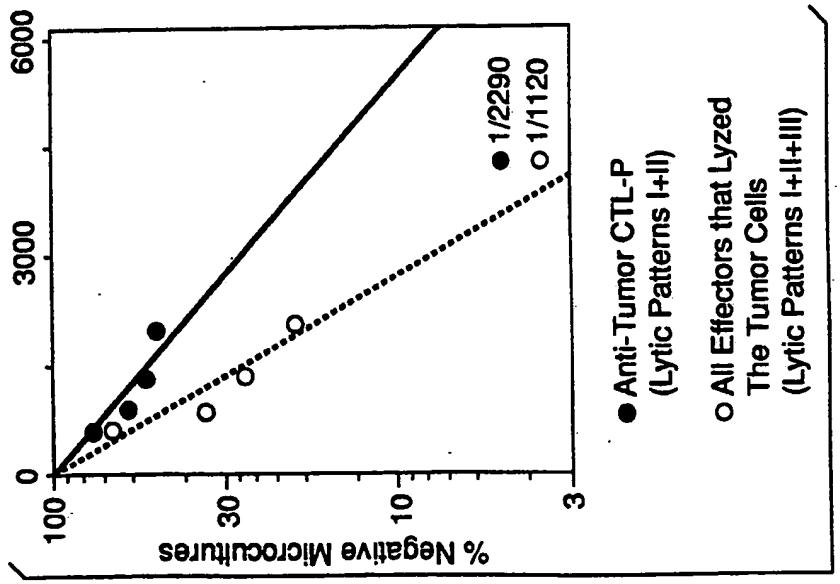


FIG. 5B

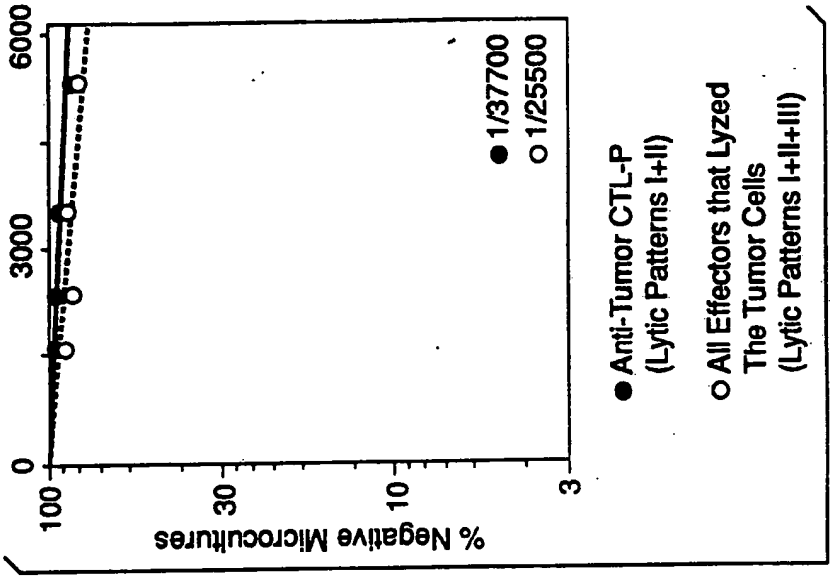
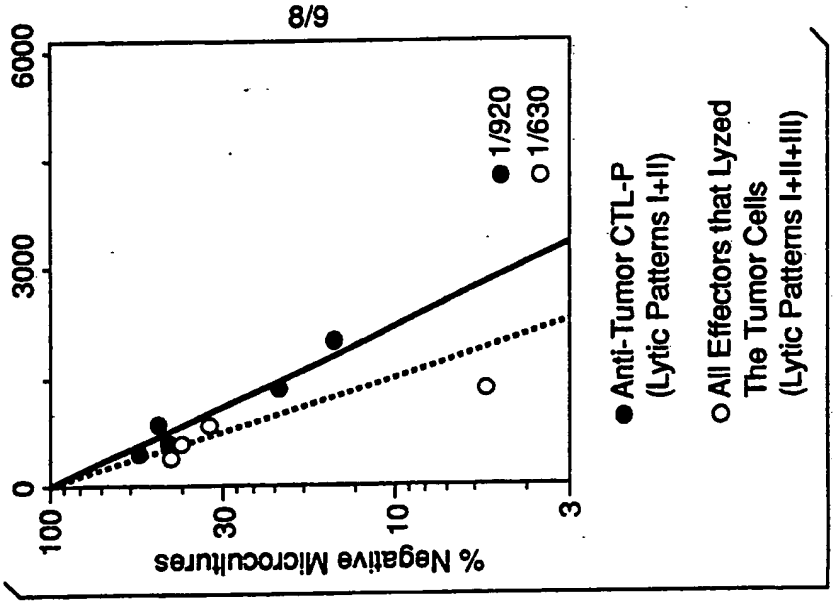


FIG. 5C



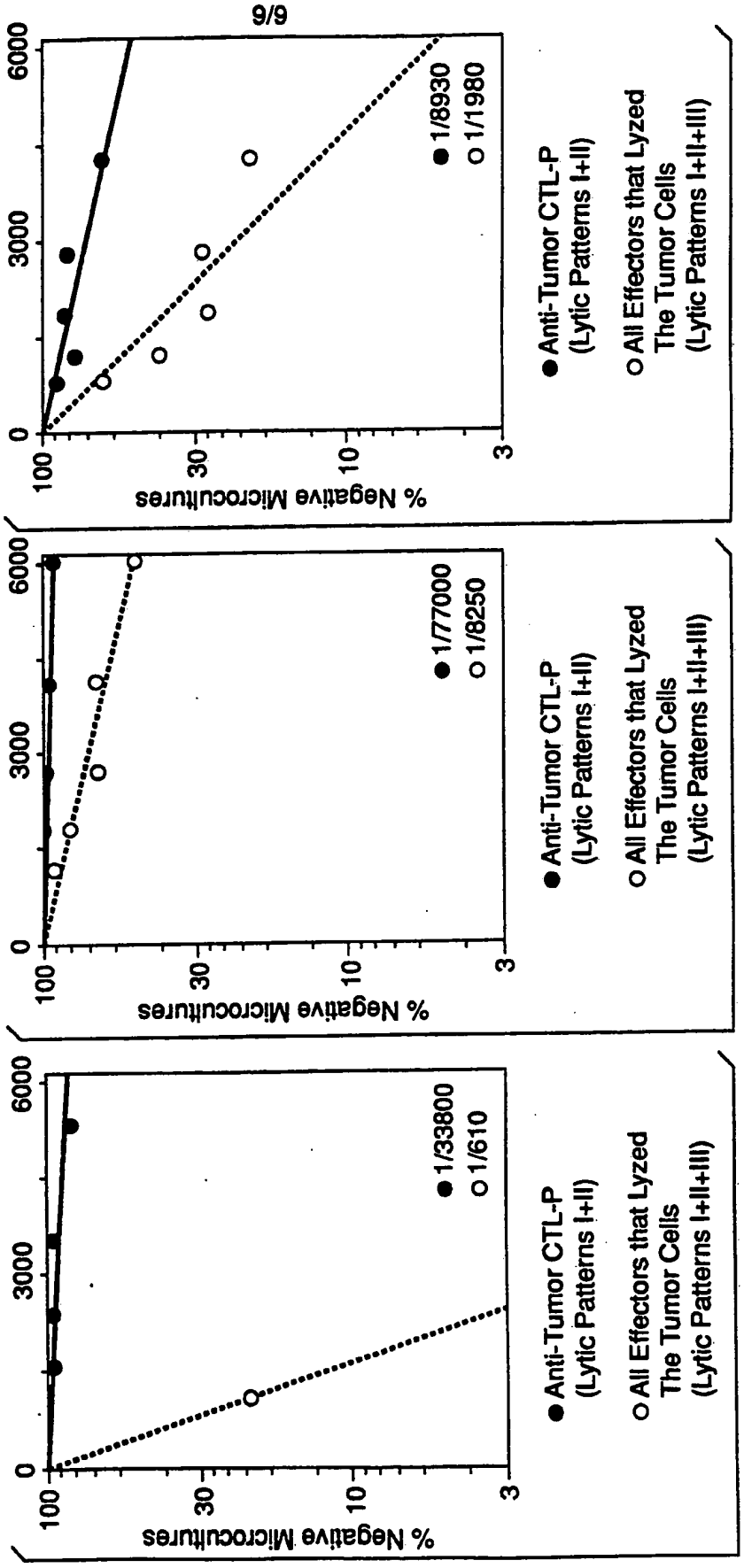


FIG. 5D
FIG. 5E
FIG. 5F
% Negative Microcultures
100
30
10
3
0
3000
6000
● 1/33800
○ 1/610
● 1/77000
○ 1/8250
● 1/8930
○ 1/1980
● Anti-Tumor CTL-P (Lytic Patterns I+II)
○ All Effectors that Lyzed The Tumor Cells (Lytic Patterns I+II+III)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00083

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/00; G01N 33/48, 37/00
US CL :435/7.24, 240.21, 240.25; 436/56, 57, 64
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.24, 240.21, 240.25, 962; 436/56, 57, 64, 804, 813

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: cytolytic T cell precursor, tumor rejection antigen, tumor antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	INTERNATIONAL JOURNAL OF CANCER, Volume 44, issued 1989, B. Van Den Eynde et al, "Presence on a Human Melanoma of Multiple Antigens Recognized by Autologous CTL", pages 634-640, especially pages 635-637.	1,3-9,12,13 2,10,11,14, 15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 February 1993	Date of mailing of the international search report 04 MAR 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer NANCY J. PARSONS Telephone No. (703) 308-0196